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(54) BETA-FRUCTOFURANOSIDASE AND GENE THEREOF

(57) A novel β-fructofuranosidase and its gene are disclosed. A polypeptide comprising the amino acid sequence of SEQ ID No. 1 or No. 3 is an enzyme having β-fructofuranosidase activity and high transferase activity, and is capable of efficiently producing fructooligosaccharides.

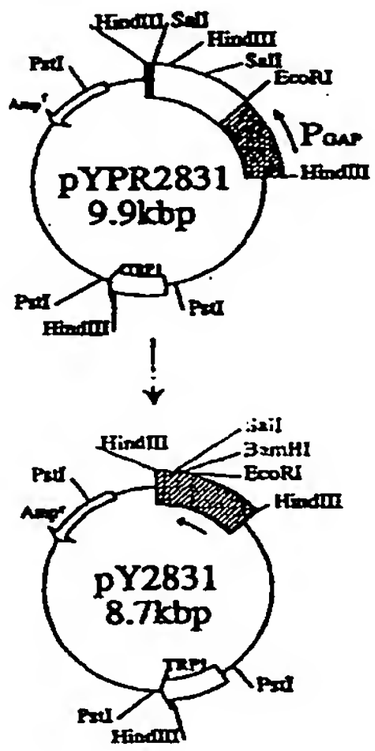

FIG. 1A

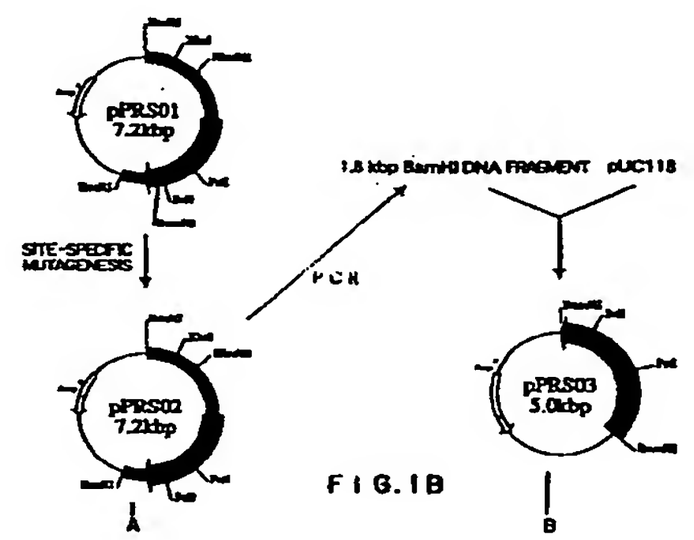

FIG. 1B

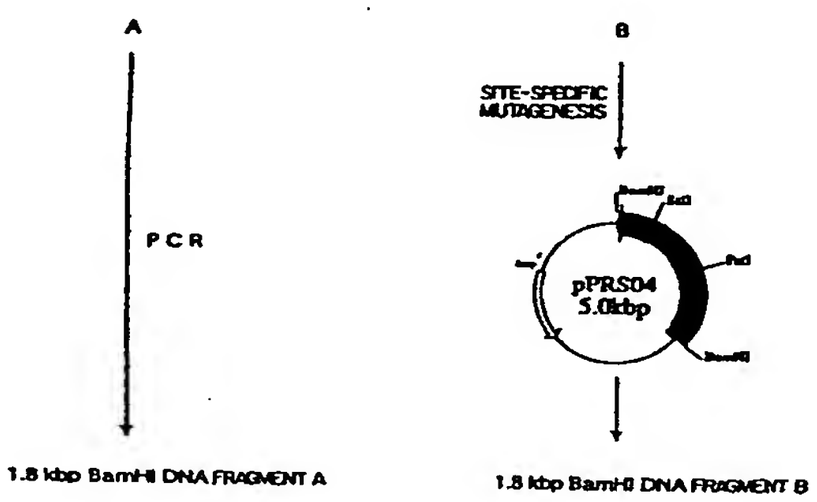

FIG. 1C

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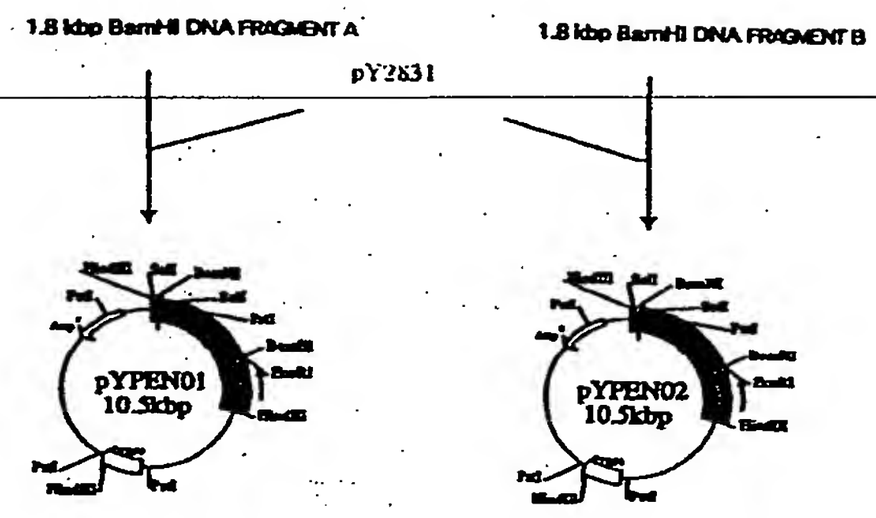

FIG. 1D

Description

Background of the Invention

Field of the Invention

[0001] The present invention relates to a β-fructofuranosidase having a fructose transferase activity, which is useful for the industrial production of fructooligosaccharides, and its gene and use.

Background Art

[0002] The molecular structure of a fructooligosaccharide is the same as that of sucrose, except that the fructose half of a fructooligosaccharide is coupled with another one to three fructose molecules at positions C1 and C2 via a β-bond. Fructooligosaccharides are indigestible sugars known for their physiological advantages, such as the facilitation of Bifidobacterial growth in the intestines, metabolic stimulation for cholesterols and other lipids, and little cariosity.

[0003] Fructooligosaccharides are found in plants, such as asparagus, onion, Jerusalem-artichoke and honey. They are also synthesized from sucrose by the newly industrialized mass production technique using fructosyltransfer reaction which is catalyzed by a β-fructofuranosidase derived from a microorganism.

[0004] The molecular structure of 1-kestose and nystose, which make up component of industrially produced fructooligosaccharide mixtures of today, are the same as that of sucrose except that their fructose half is coupled with one and two molecules of fructose, respectively. It has been found recently that their high-purity crystals exhibit new desirable characteristics both in physical properties and food processing purpose while maintaining the general physiological advantages of fructooligosaccharides (Japanese Patent Application No. 222923/1995, Japanese Laid-Open Publication No. 31160/1994). In this sense, they are fructooligosaccharide preparations having new features.

[0005] In consideration of the above, some of the inventors have already proposed an industrial process for producing crystal 1-ketose from sucrose (Japanese Patent Application No. 64682/1996, Japanese Patent Application No. 77534/1996, and Japanese Patent Application No. 77539/1996). According to this process, a β-fructofuranosidase harboring fructosyltransferase activity is first allowed to act on sucrose to produce 1-kestose; the resultant 1-kestose is fractionated to a purity of 80% or higher by chromatographic separation; then, using this fraction as a crystallizing sample, crystal 1-kestose is obtained at a purity of 95% or higher. The β-fructofuranosidase harboring fructosyltransferase activity used in this process should be able to produce 1-kestose from sucrose at a high yield while minimizing the byproduct nystose, which inhibits the reactions in the above steps of chromatographic separation and crystallization. In the enzyme derived from Aspergillus niger, which is currently used for the industrial production of fructooligosaccharides mixtures, the 1-kestose yield from sucrose is approximately 44%, while 7% is turned to nystose (Japanese Patent Application No. 64682/1996). These figures suggest that the enzyme has room for improvement in view of the industrial production of crystal 1-ketose.

[0006] As a next step, some of the inventors have successfully screened new enzymes having more favorable characteristics from Penicillium roqueforti and Scopulariopsis brevicaulis. These enzymes were able to turn 47% and 55% of sucrose into 1-kestose, respectively, and 7% and 4% to nystose (Japanese Patent Application No. 77534/1996, and Japanese Patent Application No. 77539/1996). These enzymes are inferior in productivity and stability to the enzyme derived from Aspergillus niger, and have room for improvement in view of the industrial production of crystal 1-ketose.

[0007] Thus, some of the inventors had paid attention to the procedure of genetic engineering as a process for improving the productivity of the enzyme, isolated the gene encoding β-fructofuranosidase from Penicillium roqueforti and Scopulariopsis brevicaulis, respectively, and conducted the structure analysis (PCT/JP97/00757). As a result, the translation regions encoding 565 amino acids and 574 amino acids as a mature protein were respectively deduced in the β-fructofuranosidase genes from Penicillium roqueforti and Scopulariopsis brevicaulis and their expression products were shown to have β-fructofuranosidase activity, like the β-fructofuranosidase gene from Aspergillus niger (L.M. Boddy et al., Curr. Genet., 24, 60-66 (1993)).

Summary of the Invention

[0008] The inventors have now found that the addition of 38 and 39 amino acids to the C-terminal of the β-fructofuranosidase genes from Penicillium roqueforti and Scopulariopsis brevicaulis, which were previously found by some of the inventors, improves its activity.

[0009] Thus, an object of the present invention is to provide a novel β-fructofuranosidase and its gene.

[0010] The novel β-fructofuranosidase according to the present invention is a polypeptide comprising the amino acid sequence of SEQ ID No. 1 or 3 or a homologue thereof.

[0011] Furthermore, the gene according to the present invention is a DNA encoding the above polypeptide.

[0012] The amino acid sequence of SEQ ID No. 1 or 3 according to the present invention is constructed by adding 38 and 39 amino acids to the C-terminals of the β-fructofuranosidase genes from Penicillium roqueforti and Scopulariopsis brevicaulis, which were previously found by some of the inventors as described above. It has been found that an intron actually exists at the region of the β-fructofuranosidase gene, which was presumed to encode the C-terminal amino acids by some of the present inventors and that the β-fructofuranosidase genes further encode 38 and 39 amino acids of the C-terminal. Surprisingly, the β-fructofuranosidase activity was remarkably improved by adding these amino acids to the C-terminal, as compared with the protein to which these sequences are not added.

Brief Description of the Drawing

[0013]

Figures 1A, B, C and D show the construction of expression vector pYPEN02 in which a gene encoding the enzyme protein consisting of the amino acid sequence of SEQ ID No. 1 is introduced, and expression vector pYPEN01 in which a gene encoding the enzyme protein consisting of the amino acid sequence from 1 to 565 of amino acid sequence of SEQ ID No. 1 is introduced.

Figures 2A and B show the construction of expression vector pYSCOP02 in which a gene encoding the enzyme protein consisting of the amino acid sequence of SEQ ID No. 3 is introduced, and expression vector pYSCOP01 in which a gene encoding the enzyme protein consisting of the amino acid sequence from 1 to 574 of amino acid sequence of SEQ ID No. 3 is introduced.

Detailed Description of the Invention

β-fructofuranosidase

[0014] The polypeptide according to the present invention comprises the amino acid sequence of SEQ ID No. 1 or 3. This polypeptide having the amino acid sequence of SEQ ID No. 1 or 3 has enzymatic activity as β-fructofuranosidase. The polypeptide according to the present invention involves a homologue of the amino acid sequence of SEQ ID No. 1 or 3 as shown in the sequence listing. The term "homologue" refers to an amino acid sequence in which one or more amino acids (for example, one to several amino acids) are inserted, substituted or deleted in, or added to either or both of the terminals of, the amino acid sequence of SEQ ID Nos. 1 and 3 while retaining β-fructofuranosidase activity. Such a homologue can be selected and produced by those skilled in the art without undue experiments by referring to the sequence of SEQ ID No. 1 or 3.

[0015] The β-fructofuranosidase having the amino acid sequence of SEQ ID Nos. 1 and 3 according to the present invention has high fructosyltransferase activity and efficiently produces fructooligosaccharides. Specifically, when a sucrose solution at a concentration of 30 wt% or more is used as a substrate for reaction, the fructosyltransferase activity of β-fructofuranosidase having the amino acid sequence of SEQ ID No. 1 is at least 4 times higher, and the fructosyltransferase activity of β-fructofuranosidase having the amino acid sequence of SEQ ID No. 3 is at least 7 times higher than hydrolytic activity. Furthermore, 50% or more of sucrose is converted to fructooligosaccharides in both cases.

β-fructofuranosidase gene

[0016] The novel gene encoding β-fructofuranosidase according to the present invention comprises a DNA sequence encoding the amino acid sequence of SEQ ID Nos. 1 and 3 or a homologue thereof.

[0017] Generally, a nucleotide sequence which encodes the amino acid sequence of a given protein can be easily determined from the reference chart known as "codon table". A variety of nucleotide sequence are available from those encoding the amino acid sequence of SEQ ID No. 1 or 3. Therefore, the term "a nucleotide sequence encoding the amino acid sequence of SEQ ID No. 1 or 3" refers to the meaning including the nucleotide sequence of SEQ ID No. 2 or 4, as well as nucleotide sequences which consist of the same codons as above allowing for degeneracy and encode the amino acid sequence of SEQ ID No. 1 or 3.

[0018] A preferred embodiment of the present invention provides, as a preferred example of the novel gene according to the present invention, a DNA fragment comprising the nucleotide sequence of SEQ ID No. 2 or 4.

[0019] As described above, the present invention encompasses a homologue of the amino acid sequence of SEQ ID No. 1 or 3. Therefore, the DNA fragment according to the present invention involves a nucleotide sequence which encodes such a homologue.

[0020] As the nucleotide sequence of the DNA fragment according to the present invention is determined, the DNA fragment may be obtained according to the procedure for the synthesis of a nucleic acid.

[0021] This sequence can also be obtained from Penicillium roqueforti or Scopulariopsis brevicaulis, preferably

Penicillium roqueforti IAM7254 or Scopulariopsis brevicaulis IFO4843, according to the procedure of genetic engineering.

Expression of β-fructofuranosidase Gene

[0022] The β-fructofuranosidase according to the present invention can be produced in a host cell which has been transformed by a DNA fragment encoding the enzyme. More specifically, a DNA fragment encoding the β-fructofuranosidase according to the present invention is introduced in a host cell in the form of a DNA molecule which is replicatable in the host cell and can express the above gene, particularly an expression vector, in order to transform the host cell. Then, the obtained transformant is cultivated.

[0023] Therefore, the present invention provides a DNA molecule which comprises a gene encoding the β-fructofuranosidase according to the present invention, particularly an expression vector. This DNA molecule is obtained by introducing a DNA fragment encoding the β-fructofuranosidase according to the present invention in a vector molecule. According to a preferred embodiment of the present invention, the vector is a plasmid.

[0024] The DNA molecule according to the present invention may be prepared by the standard technique of genetic engineering.

[0025] The vector applicable in the present invention can be selected as appropriate from viruses, plasmids, cosmid vectors, etc., considering the type of the host cell used. For example, a bacteriophage in the λ phage group or a plasmid in the pBR or pUC group may be used for E. coli host cells, a plasmid in the pUB group for Bacillus subtilis, and a vector in the YEp or YCp group for yeast.

[0026] It is preferable that the plasmid contain a selectable marker to ensure the selection of the obtained transformance, such as a drug-resistance marker or marker gene complementing an auxotrophic mutation. Preferred example of marker genes include ampicillin-resistance gene, kanamycin-resistance gene, and tetracycline-resistance gene for bacterium host cells; N-(5'-phosphoribosyl)-anthranilate isomerase gene (TRP1), orotidine-5'-phosphate decarboxylase gene (URA3), and β-isopropylmalate dehydrogenase gene (LEU2) for yeast; and hygromycin-resistance gene (hph), bialophos-resistance gene (bar), and nitrate reductase gene (niaD) for mold.

[0027] It is also preferable that the DNA molecule for use as an expression vector according to the present invention contain nucleotide sequence necessary for the expression of the β-fructofuranosidase gene, including transcription and translation control signals, such as a promoter, a transcription initiation signal, a ribosome binding site, a translation termination signal, and a transcription termination signal.

[0028] Examples of preferred promoters include, in addition to the promoter on the inserted fragment which is able to function in the host, promoters such as those of lactose operon (lac), and tryptophan operon (trp) for E. coli; promoters such as those of alcohol dehydrogenase gene (ADH), acid phosphatase gene (PHO), galactose regulated gene (GAL), and glyceraldehyde-3-phosphate dehydrogenase gene (GPD) for yeast; and promoters such as those of α-amylase gene (amy) and cellobiohydrolase I gene (CBHI) for mold.

[0029] When the host cell is Bacillus subtilis, yeast or mold, it is also advantageous to use a secretion vector to allow it to extracellularly secrete the produced recombinant β-fructofuranosidase. Any host cell with an established host-vector system may be used, preferably yeast, mold, etc. It is preferable also to use the mold fungus having no β-fructofuranosidase activity described in PCT/JP97/00757.

[0030] A novel recombinant enzyme produced by the transformant described above is obtained by the following procedure: first, the host cell described above is cultivated under suitable conditions to obtain the supernatant or cell bodies from the resultant culture, using a known technique such as centrifugation; cell bodies should be further suspended in a suitable buffer solution, then homogenized by freeze-and-thaw, ultrasonic treatment, or mortar, followed by centrifugation or filtration to separate a cell body extract containing the novel recombinant enzyme.

[0031] The enzyme can be purified by combining the standard techniques for separation and purification. Examples of such as techniques include processes such as heat treatment, which rely on the difference in thermal resistance; processes such as salt sedimentation and solvent sedimentation, which rely on the difference in solubility; processes such as dialysis, ultrafiltration and gel filtration, and SDS-polyacrylamide gel electrophoresis, which rely on the difference in molecular weight; processes such as ion exchange chromatography, which rely on the difference in electric charge; processes such as affinity chromatography, which rely on specific affinity; processes such as hydrophobic chromatography and reversed-phase partition chromatography, which rely on the difference in hydrophobicity; and processes such as isoelectric focusing, which rely on the difference in isoelectric point.

Production of fructooligosaccharides using the β-fructofuranosidase

[0032] The present invention further provides a process for producing fructooligosaccharide using the recombinant host or recombinant β-fructofuranosidase described above.

[0033] In the process for producing fructooligosaccharides according to the present invention, the recombinant host

or recombinant β-fructofuranosidase described above is brought into contact with sucrose.

[0034] The mode and conditions where the recombinant host or recombinant β-fructofuranosidase according to the present invention comes in contact with sucrose are not limited in any way provided that the novel recombinant enzyme is able to act on sucrose. A preferred embodiment for contact in solution is as follows: The sucrose concentration may be selected as appropriate in the range where sucrose can be dissolved. However, considering the conditions such as the specific activity of the enzyme and reaction temperature, the concentration should generally fall in the range of 5% to 80%, preferably 30% to 70%. The temperature and pH for the reaction of sucrose by the enzyme should preferably be optimized for the characteristics of the novel recombinant enzyme. Therefore, the reasonable conditions are about 30°C to 80°C, pH 4 to 10, preferably 40°C to 70°C, pH 5 to 7.

[0035] The degree of purification of the novel recombinant enzyme may be selected as appropriate. The enzyme may be used either as unpurified in the form of supernatant from a transformant culture or cell body homogenate, as purified after processed in various purification steps, or as isolated after processed by various purification means.

[0036] Furthermore, the enzyme may be brought into contact with sucrose as fixed on a carrier using the standard technique.

[0037] The fructooligosaccharides thus produced are purified from the resulting solution according to known procedures. For example, the solution may be heated to inactivate the enzyme, decolorized using activated carbon, then desalted using ion exchange resin.

Examples

Example 1: Determination of translation region of β-fructofuranosidase gene from Penicillium roqueforti IAM7254

[0038] A DNA fragment of about 2 kbp containing the β-fructofuranosidase gene from Aspergillus niger was amplified by PCR using a chromosomal DNA prepared from Aspergillus niger ATCC20611 according to the standard procedure as a template and synthetic DNAs of SEQ ID Nos. 5 and 6 as primers. This DNA fragment was fractionated by agarose gel electrophoresis, extracted according to the standard procedure, purified, and then dissolved in sterilized water to 0.1 μg/μl to prepare a DNA sample for probe.

[0039] In the next step, a chromosomal DNA from Penicillium roqueforti IAM7254 was prepared, about 20 μg of the chromosomal DNA was digested completely with EcoRI, followed by agarose gel electrophoretsis to recover about 4 kbp DNA fragments.

[0040] The recovered DNA fragments of about 4 kbp (about 0.5 μg) were ligated with 1 μg of λgt10 vector which had been digested with EcoRI and treated with phosphatase, packaged using an in vitro packaging kit, GIGAPACK II Gold (Stratagene L.L.C.), and then introduced in E. coli NM514, to prepare a library.

[0041] A probe was prepared from DNA sample for probe above described. As a result of plague hybridization using ECL Direct DNA/RNA Labelling & Detection System (Amersham International), 4 clones turned out positive in about 25,000 plaques. These positive clones were purified by a second screening to prepare phage DNA, which was then analyzed using restriction enzymes. The result showed that all the clones had an identical EcoRI fragment of about 4 kbp.

[0042] The EcoRI fragments of about 4 kbp were subdivided into a small fragment to select the desired DNA region using restriction enzymes, then subcloned to plasmid vector pUC118 or pUC119. The plasmid DNA was obtained from the subclone according to the standard procedure and sequenced using ALFred DNA Sequencer (Pharmacia) as shown in SEQ ID No.7.

[0043] The sequence consisting 50 bases from 1695 to 1744 in this sequence was identified as an intron because it showed a typical intron structure of filamentous fungi. As a result, the sequence of SEQ ID No. 2 as a sequence encoding protein was obtained by deleting the intron from the sequence of SEQ ID No. 7. The encoded amino acid sequence was shown in SEQ ID No. 1.

Example 2: Expression of β-fructofuranosidase gene from Penicillium roqueforti IAM7254 in Saccharomyces cerevisiae

[0044] Plasmid pYPEN01 and pYPEN02 for expressing the β-fructofuranosidase gene from Penicillium roqueforti were prepared as follows (Figure 1A, B, C and D).

[0045] pYPR2831 (H. Horiuchi et al., Agric. Biol. Chem., 54, 1771-1779, 1990) was digested with EcoRI and SalI, and then its terminals were blunted with T4 DNA polymerase. The obtained fragment was ligated with BamHI linker (5'-CGGATCCG-3'), digested with BamHI, followed by self-ligation to obtain vector pY2831 for expression in yeast.

[0046] Next, single-stranded DNA was prepared from the plasmid pPRS01 obtainad by inserting an about 4 kbp EcoRI DNA fragment containing the β-fructofuranosidase gene prepared in Example 1 into plasmid pUC118. Using the single-stranded DNA as a template and a synthetic DNA of SEQ ID No. 8 as a primer, the translated region of the β-fructofuranosidase gene was subjected to site-specific mutagenesis to disrupt the BamHI site without changing the

encoded amino acid sequence (pPRS02).

[0047] A part of the translated region of the β-fructofuranosidase gene was prepared as an about 1.8 kbp BamHI fragment by PCR using plasmid pPRS02 as a template and synthetic DNAs of SEQ ID Nos. 9 and 10 as primers, and inserted into the BamHI site of plasmid pY2831 to prepare pYPEN01. Thus, plasmid pYPEN01 is designed to produce an enzyme protein comprising an amino acid sequence from 1 to 565 in the amino acid sequence of SEQ ID No. 1, which is a mature β-fructofuranosidase following secretion signal sequence.

[0048] Further, a DNA fragment containing the translated region of the β-fructofuranosidase gene was prepared as an about 1.8 kbp BamHI fragment by PCR using plasmid pPRS02 as a template and synthetic DNAs of SEQ ID Nos. 9 and 11 as primers, and inserted into the BamHI site of plasmid pUC118 to prepare plasmid pPRS03. A single-stranded DNA was prepared from plasmid pPRS03. As a result of site-specific mutagenesis using this as a template and a synthetic DNA of SEQ ID No. 12 as a primer, the intron sequence was removed (pPRS04). The translated region of the β-fructofuranosidase gene was prepared as an about 1.8 kbp BamHI fragment from plasmid pPRS04, and inserted into the BamHI site of plasmid pY2831 to prepare plasmid pYPEN02. Thus, plasmid pYPEN02 is designed to produce an enzyme protein comprising an amino acid sequence of SEQ ID No. 1, which is a mature β-fructofuranosidase following secretion signal sequence.

[0049] Plasmids pYPEN01 and pYPEN02 were introduced into Saccharomyces cerevisiae MS-161 (Suc$^-$, ura3, trp1) by the lithium-acetate method (Ito, H. et al., J. Bacteriol., 153, 163-168, 1983) to obtain transformants. The transformants were cultivated in an SD-Ura medium (0.67% yeast nitrogen base (Difco), 2% glucose and 50μg/ml uracil) at 30°C overnight. The culture was seeded in a production medium (0.67% yeast nitrogen base Difco, 2% glucosece, 2% casamino acid and 50μg/ml uracil) at a final concentration of 1% and cultivated at 30°C for 2 days. The culture supernatant was analyzed for β-fructofuranosidase activity, in units, i.e., the quantity of free glucose (μ mol) released in 1 minute in 10 wt% sucrose solution, pH 5.5, at 40°C for 60 minutes. As a result, the transformant with plasmid pYREN01 exhibited 4 x 10^{-4} units/ml or less of activity while the transformant with plasmid pYREN02 exhibited 0.38 units/ml of activity.

Example 3: Determination of the translated region of β-fructofuranosidase gene from Scopulariopsis brevicaulis IFO4843

[0050] The chromosomal DNA was prepared from Scopulariopsis brevicaulis IFO4843. About 20 μg of a chromosomal DNA sample was completely digested with EcoRI, and electrophoresed through an agarose gel to recover an about 10 kbp DNA fragment.

[0051] The recovered DNA fragment of about 10 kbp (about 0.5 μg) were ligated with 1 μg of λDASHII vector digested with HindIII and EcoRI, and packaged using an in vitro packaging kit, GIGAPACK II Gold (Stratagene L.L.C.), then introduced in E. coli XL1-Blue MRA (P2), to prepare a library.

[0052] As a result of plague hybridization using ECL Direct DNA/RNA Labelling & Detection System (Amersham International), with the about 2 kbp DNA fragment used in Example 1 as a probe, 3 clones turned out positive in about 15,000 plaques. These positive clones were purified by a second screening to prepare phage DNA, which was then analyzed using restriction enzymes. The result showed that all the clones had an identical EcoRI fragment of about 10 kbp.

[0053] These EcoRI fragments of about 10 kbp were subdivided into a small fragment to select the desired DNA region using restriction enzymes, then subcloned to plasmid vector pUC118 or pUC119. The plasmid DNA was obtained from the subclone according to the standard procedure and sequenced using ALFred DNA Sequencer (Pharmacia) as shown in SEQ ID No.13.

[0054] The sequence comprising 55 bases from 1722 to 1776 in this sequence was identified as an intron because it showed a typical intron structure of filamentous fungi. As a result, the sequence of SEQ ID No. 4 as a sequence encoding protein was obtained by deleting the intron from the sequence of SEQ ID No. 13. The encoded amino acid sequence was shown SEQ ID No. 3.

Example 4: Expression of β-fructofuranosidase gene from Scopulariopsis brevicaulis IFO4843 in Saccharomyces cerevisiae

[0055] Plasmid pYSCOP01 and pYSCOP02 for expressing the β-fructofuranosidase gene from Scopulariopsis brevicaulis were prepared as follows (Figure 2A and B).

[0056] A part of the translated region of the β-fructofuranosidase gene was prepared as an about 1.8 kbp BamHI fragment by PCR using about 10 kbp EcoRI DNA fragment prepared in Example 3 containing the β-fructofuranosidase gene as a template and synthetic DNAs of SEQ ID Nos. 14 and 15 as primers, and inserted into the BamHI site of plasmid pY2831 to prepare pYSCOP01. Thus, plasmid pYPEN01 is designed to produce an enzyme protein comprising an amino acid sequence from 1 to 574 in the amino acid sequence of SEQ ID No. 3, which is a mature β-fructofuranosi-

dase following secretion signal sequence.

[0057] Next, a DNA fragment containing the translated region of the β-fructofuranosidase gene was prepared as an about 1.9 kbp BamHI fragment by PCR using an about 10 kbp EcoRI fragment containing the β-fructofuranosidase gene as a template and synthetic DNAs of SEQ ID Nos. 14 and 16 as primers, and inserted into the BamHI site of plasmid pUC118 to prepare plasmid pSCB01. A single-stranded DNA was prepared from plasmid pSCB01. As a result of site-specific mutagenesis using this as a template and the synthetic DNA of SEQ ID No. 17 as a primer, the intron sequence was removed (pSCB02). The translated region of the β-fructofuranosidase gene was prepared as an about 1.9 kbp BamHI fragment from plasmid pSCB02, and inserted into the BamHI site of plasmid pY2831 to prepare plasmid pYSCOP02. Thus, plasmid pYSCOP02 is designed to produce an enzyme protein comprising an amino acid sequence of SEQ ID No. 3, which is a mature β-fructofuranosidase following secretion signal sequence.

[0058] Plasmids pYSCOP01 and pYSCOP02 were introduced into Saccharomyces cerevisiae MS-161 (Suc$^-$, ura3, trp1) by the lithium-acetate method to obtain transformants. The transformants were cultivated in an SD-Ura medium at 30°C overnight. The culture was seeded a production medium at a final concentration of 1% and cultivated at 30°C for 2 days. The culture supernatant was analyzed for β-fructofuranosidase activity in the same manner as described in Example 2. As a result, the transformant with plasmid pYSCOP01 exhibited 4×10^{-4} units/ml or less of activity, while the transformant with plasmid pYSCOP02 exhibited 6.5×10^{-3} units/ml of activity.

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275 280 285

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Phe Lys Pro Thr Met Ala Gly Val Leu Asp Trp Gly Val Ser Ala Tyr

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Ala Ala Ala Gly Lys Ile Leu Pro Ala Ser Ser Gln Ala Ser Thr Lys

325 330 335

Ser Gly Ala Pro Asp Arg Phe Ile Ser Tyr Val Trp Leu Thr Gly Asp

340 345 350

Leu Phe Glu Gln Val Lys Gly Phe Pro Thr Ala Gln Gln Asn Trp Thr

355 360 365

Gly Ala Leu Leu Leu Pro Arg Glu Leu Asn Val Arg Thr Ile Ser Asn

370 375 380

Val Val Asp Asn Glu Leu Ser Arg Glu Ser Leu Thr Ser Trp Arg Val

385 390 395 400

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420 425 430

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Thr Ser Ala Arg Asp Ser Gly Ile Gln Ala Gly Phe Gln Val Leu Ser

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Gly Thr Trp Ala Arg Ser Trp Tyr Ala Asn Ser Thr Lys Ile Asn Phe
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Ile Gly Asp Pro Cys Met His Tyr Thr Asp Pro Glu Thr Gly Ile Phe
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His Val Gly Trp Leu Tyr Asn Gly Asn Gly Ala Ser Gly Ala Thr Thr
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Glu Asp Leu Val Thr Tyr Gln Asp Leu Asn Pro Asp Gly Ala Gln Met
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Ile Leu Pro Gly Gly Val Asn Asp Pro Ile Ala Val Phe Asp Gly Ala
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Val Ile Pro Ser Gly Ile Asp Gly Lys Pro Thr Met Met Tyr Thr Ser
115 120 125

Val Ser Tyr Met Pro Ile Ser Trp Ser Ile Ala Tyr Thr Arg Gly Ser
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Ser Ser Ala Ala Ala Leu Thr Thr Asp Gly Ile Asp Thr Arg Asn Glu
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Phe Gly Lys Met Arg Leu Phe Asp Val Val Glu Gly Asp Gln Glu Arg
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Val His Ala Asn Gly Arg Phe Ala Leu Ser Thr Trp Val Arg Ser Trp
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Tyr Glu Ser Ser Lys Asp Ile Lys Phe Phe His Asp Gly Asp Ser Thr
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Claims

1. A polypeptide comprising the amino acid sequence of SEQ ID No. 1 or a homologue thereof.

2. A DNA encoding a polypeptide according to Claim 1.

3. A DNA according to Claim 2 comprising the nucleotide sequence of SEQ ID No. 2.

4. A polypeptide comprising the amino acid sequence of SEQ ID No. 3 or a homologue thereof.

5. A DNA encoding a polypeptide according to Claim 4.

6. A DNA according to Claim 5 comprising the nucleotide sequence of SEQ ID No. 4.

7. A vector comprising a DNA according to Claim 2, 3, 5 or 6.

8. A host cell transformed by a vector according to Claim 7.

9. A process for producing a β-fructofuranosidase comprising the steps of:

cultivating a host cell according to Claim 8, and
collecting the β-fructofuranosidase from the host and/or the culture thereof.

10. A process for producing fructooligosaccharides comprising the step of bringing sucrose into contact with a host cell according to Claim 8 or a β-fructofuranosidase obtained by the process according to Claim 9.

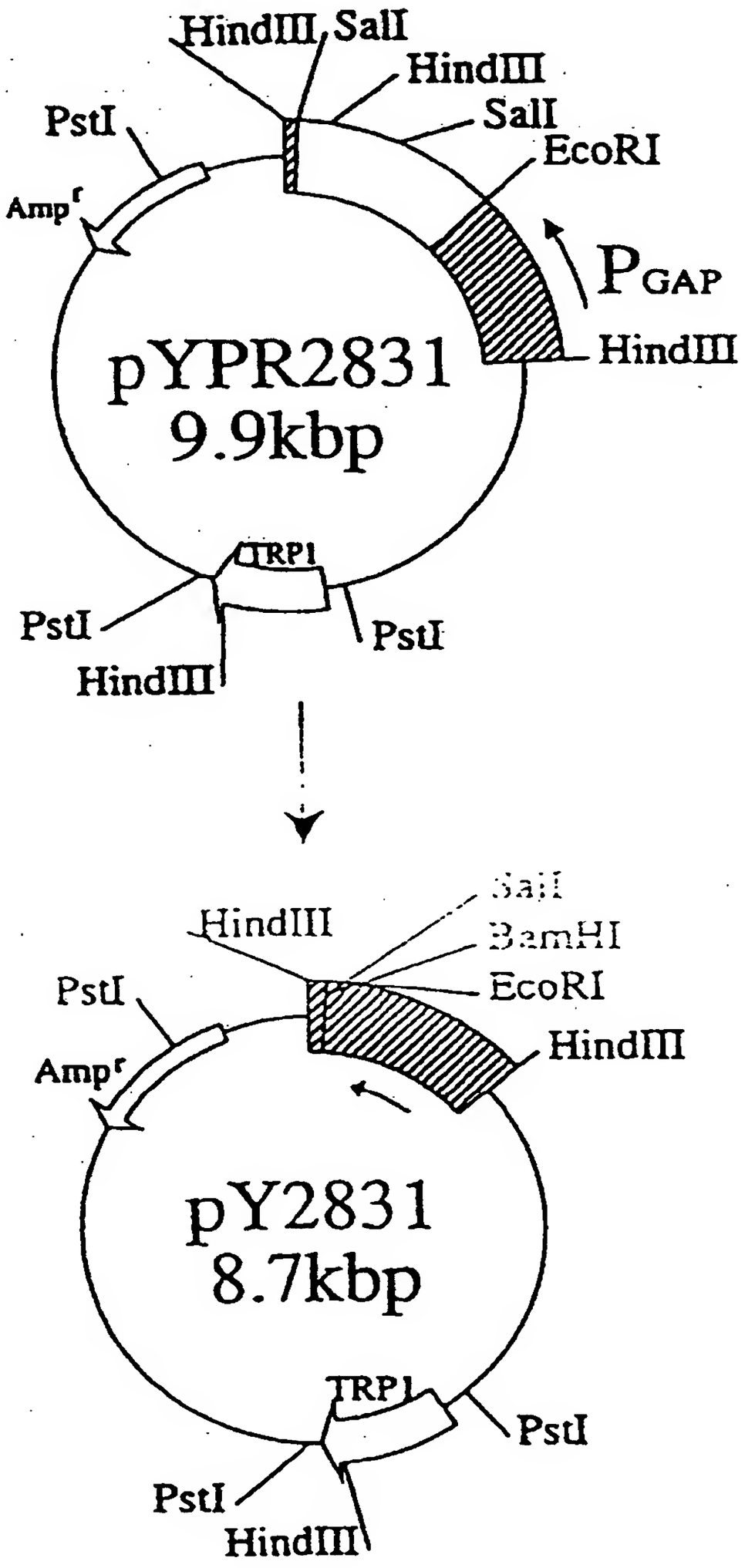

FIG. 1A

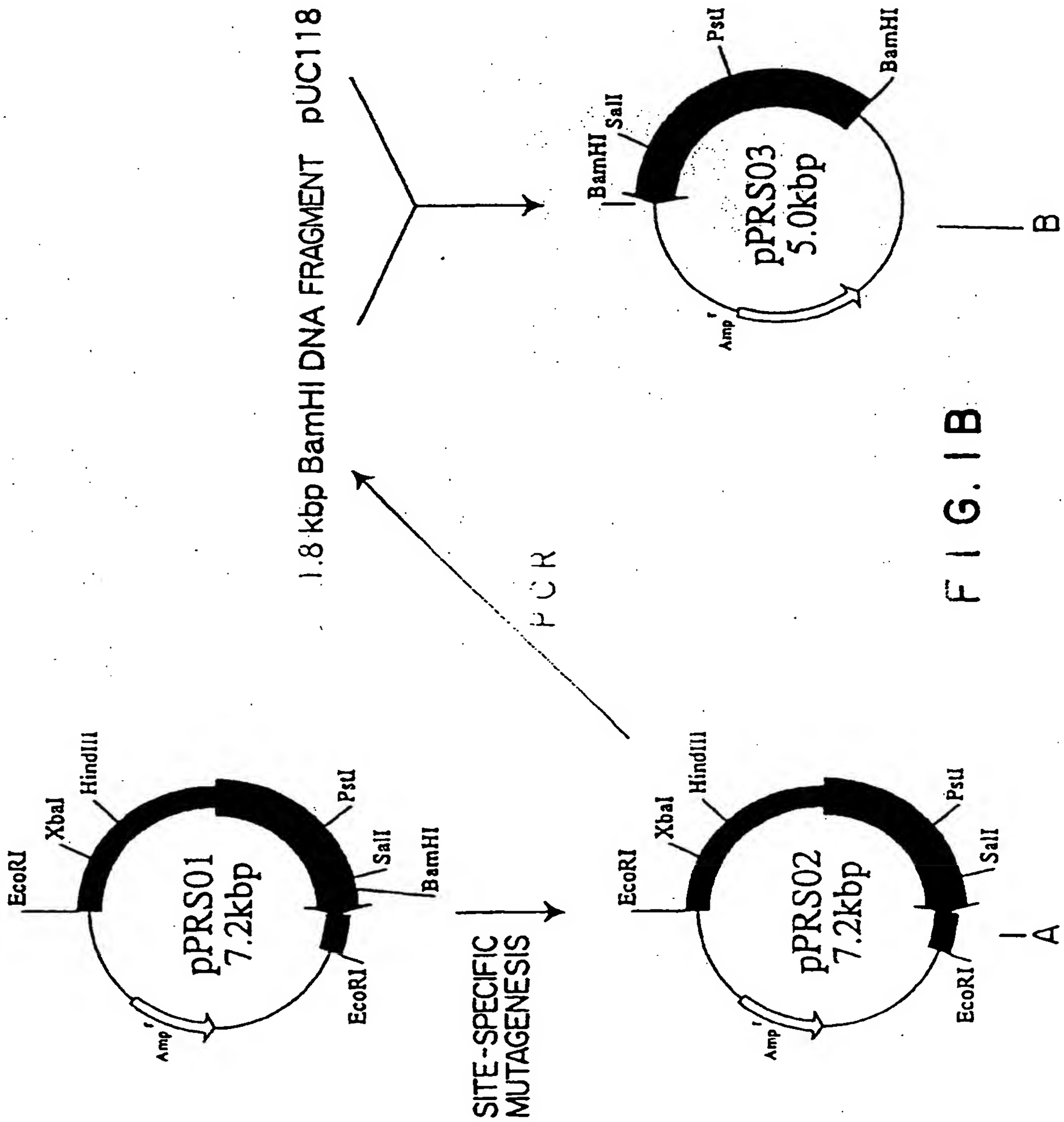

FIG. 1B

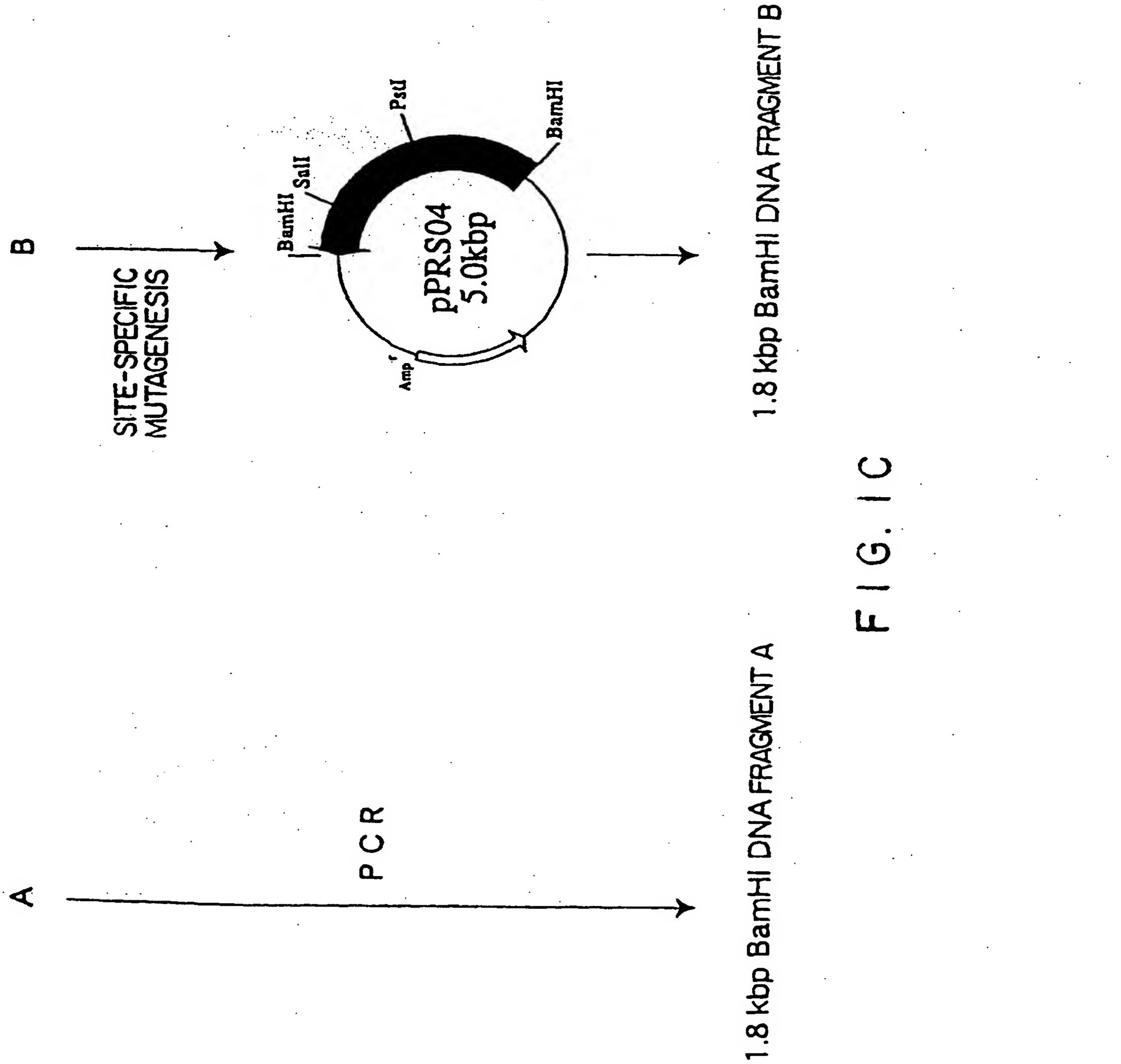
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FIG. 1C

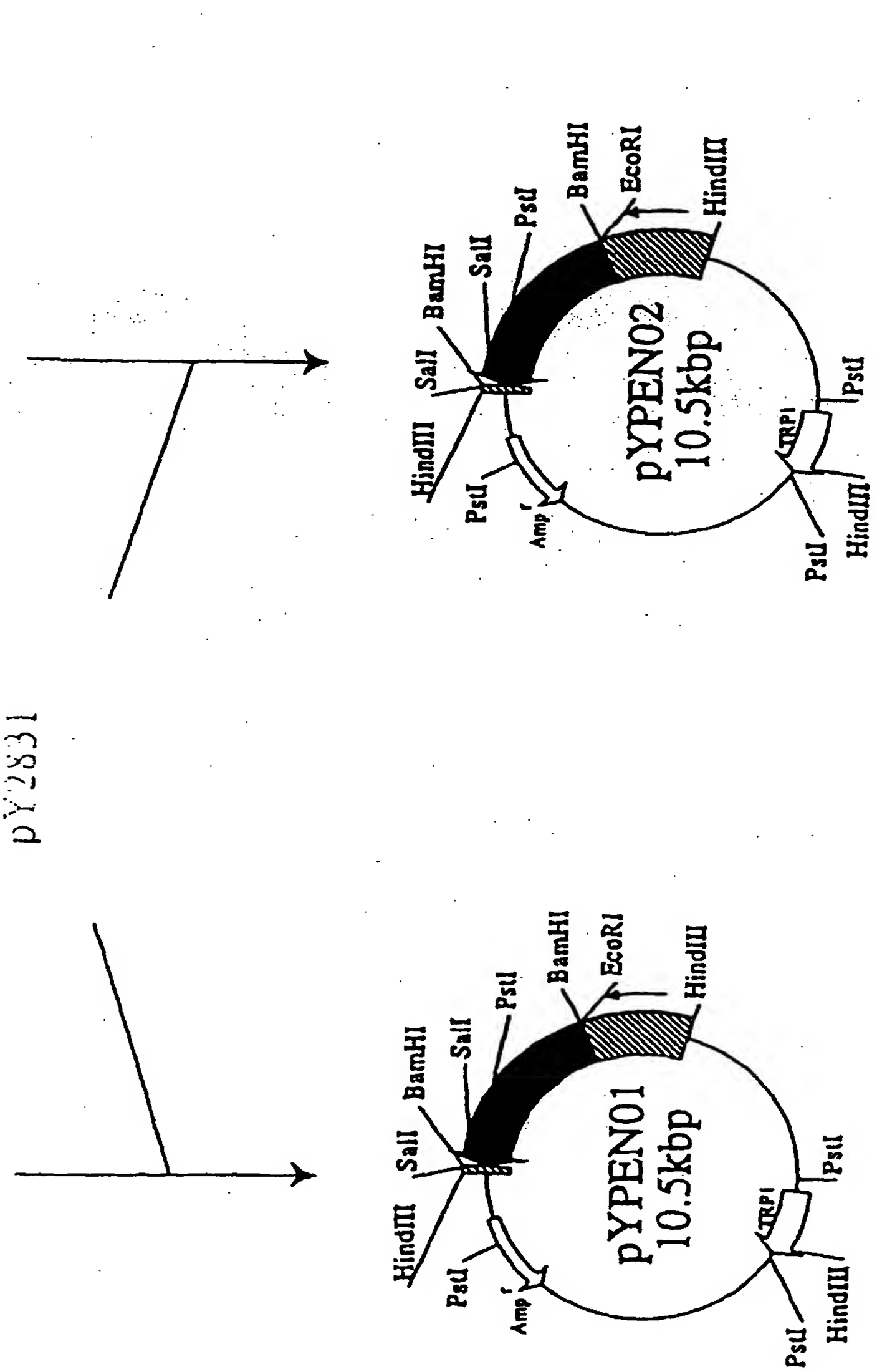

FIG. 1D

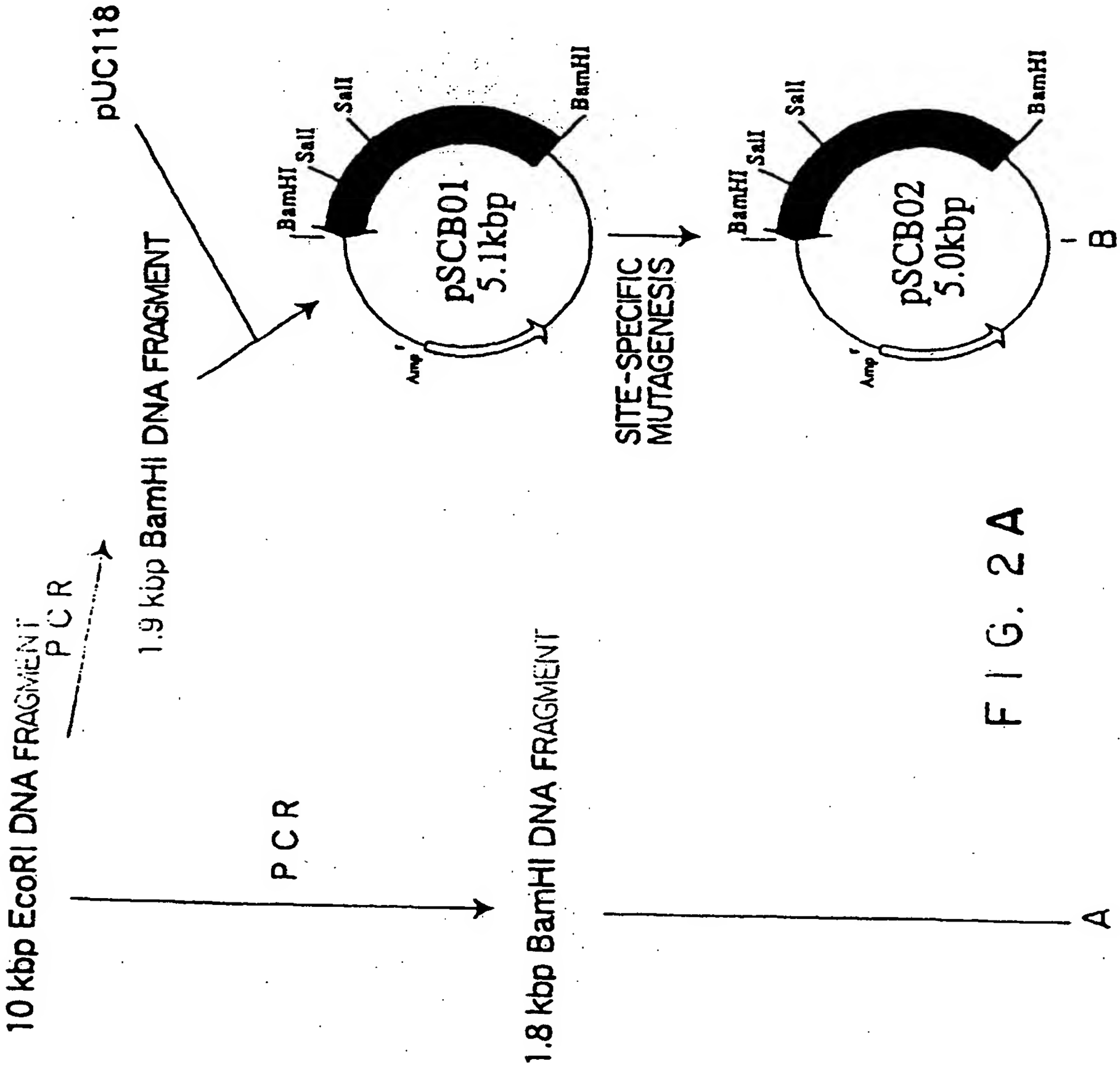
10 kbp EcoRI DNA FRAGMENT
PCR
1.9 kbp BamHI DNA FRAGMENT
pUC118
BamHI
SalI
pSCB01
5.1kbp
Amp
SITE-SPECIFIC MUTAGENESIS
pSCB02
5.0kbp
PCR
1.8 kbp BamHI DNA FRAGMENT
A
B

FIG. 2A

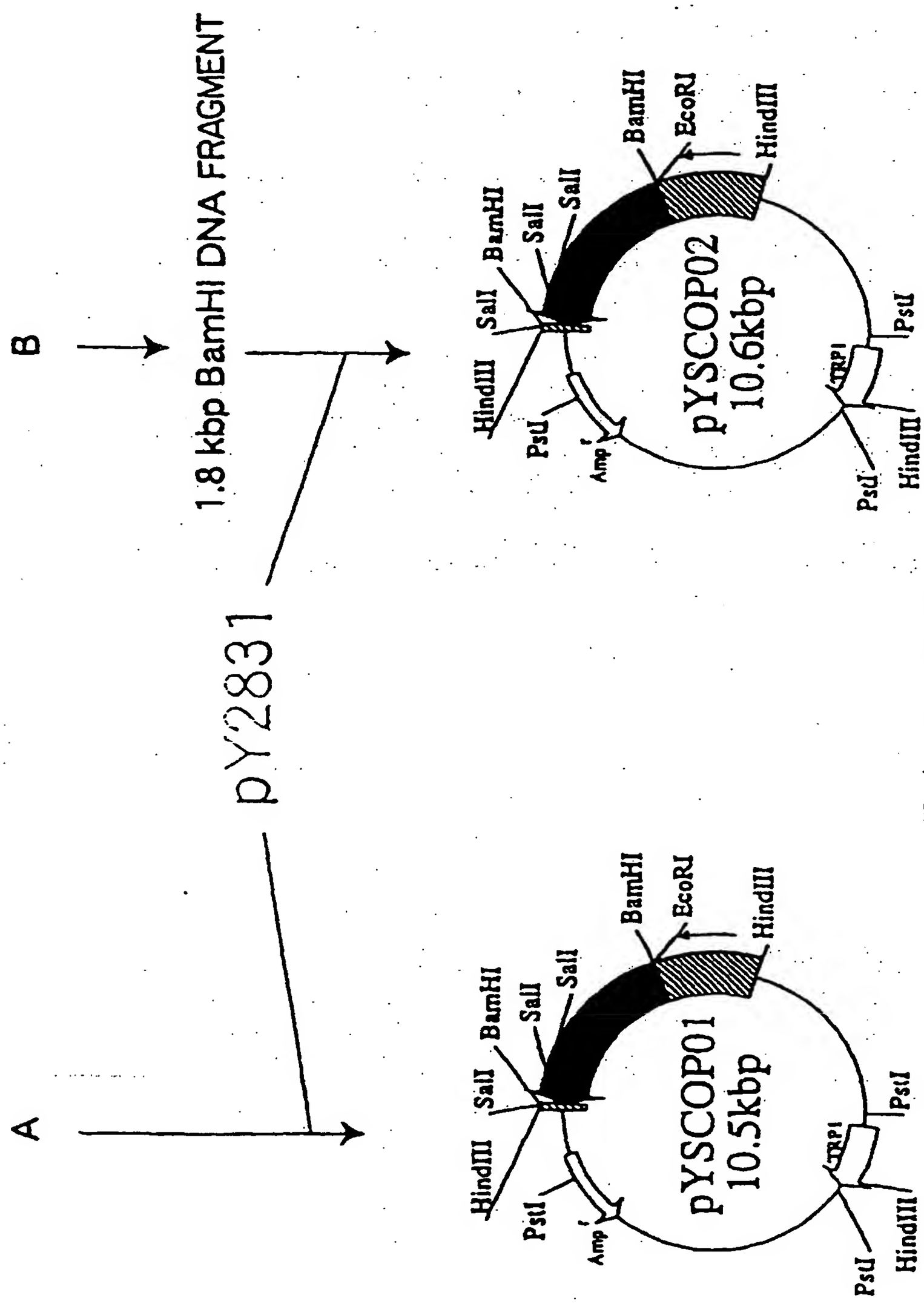

FIG. 2B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/04087

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl⁶ C12N9/10, C12N15/54, C12N1/19, C12P19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl⁶ C12N9/10, C12N15/54, C12N1/19, C12P19/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DDBJ/EMBL/GenBank

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	L.M. Boddy et al., "Purification and characterization of an Aspergillus nigar invertase and its DNA sequence", Curr. Genet., Vol. 24, P.60-66 (1993)	1-10
P, X	WO, 97/34004, A (Meiji Seika Kaisha, Ltd.), 18 September, 1997 (18. 09. 97) (Family: none)	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 November, 1998 (27. 11. 98)	8 December, 1998 (08. 12. 98)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)